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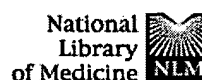
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## Characterization of the Hepatitis B Virus EnhI Enhancer and X Promoter Complex

WENTONG GUO, KENNETH D. BELL, AND JING-HSIUNG OU\*

*Department of Microbiology, University of Southern California,  
Los Angeles, California 90033-1054*

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The hepatitis B virus EnhI enhancer element overlaps the promoter of the X gene. By performing methylation interference experiments, four protein factor binding sites clustered in a 120-bp region were found to control the EnhI enhancer and X promoter activities. Deletion mapping experiments indicated that the two upstream protein factor binding sites constituted a basal enhancer module. This module, likely bound by a liver-specific factor and a ubiquitous factor, could activate the herpes simplex virus thymidine kinase gene promoter by 5- or 10-fold, depending on the orientation, in Huh7 cells, a liver-derived cell line, but not in other cell types tested. The two downstream protein factor binding sites interact with the upstream basal enhancer module in an orientation- and distance-dependent manner to increase the enhancer activity by another 10-fold. In addition, at least one of the two downstream protein factor binding sites is also essential for the X promoter activity.

Hepatitis B virus (HBV) is a small DNA virus with a genome size of about 3.2 kb. The genome of HBV contains four genes named S, C, P, and X genes. Transcription of these four genes is regulated by four different promoters and two enhancer elements. The two enhancer elements, named EnhI (22, 24) and EnhII (31), can activate HBV promoters and other heterologous promoters in *cis*. Although EnhII is believed to be liver specific, the liver specificity of EnhI has been controversial (3, 15, 22, 24, 28).

The HBV X gene product is a transcriptional transactivator (20, 23, 26). This protein can bind to at least two different proteins, the large T antigen of simian virus 40 (23) and the transcriptional factor AP2 (19). Recent results indicate that the X protein is also a novel protein kinase capable of autophosphorylation (30). Similar to findings for other eucaryotic polymerase II genes, the transcription of X mRNA is regulated by the X promoter, located in the upstream region of the X protein coding sequence (25). This X promoter overlaps the EnhI enhancer element. The structural relationship between the X promoter and EnhI, however, is unclear.

Several protein factor binding sites that may be important for regulating EnhI enhancer activity have been identified (1, 15). The site most proximal to the X gene coding sequence contains an NF1 factor binding sequence and is believed to be bound by a factor closely related to NF1 (2, 15). A second protein factor binding site, named the E site, is located adjacent to the NF1 site. This site has no enhancer activity by itself but displays a weak ubiquitous enhancer activity when oligomerized (4). The E site can be bound by several protein factors, including C/EBP (11, 16), AP1 (4), and CREB (4), and is thought to be an essential component of the EnhI enhancer (2, 4). A third protein factor binding site, named the EP site, is located upstream of the E site (1). Because binding of protein factor to this site can be inhibited by an oligonucleotide containing the binding sequence of EF-C factor, a ubiquitous protein factor that binds to the enhancer element of polyomavirus, this site is believed to be

bound by EF-C (13, 14). Several additional protein factor binding sites located upstream of the EP site have also been identified (21). These sites, however, are not very well characterized. The importance of the protein factor binding sites discussed above on the regulation of X promoter activity has not been investigated.

In an effort to investigate the liver specificity of EnhI and to understand the structural relationship between EnhI and the X promoter, we have further characterized this EnhI enhancer-X promoter complex. Our studies have allowed us to identify a new protein factor binding site. This site is likely bound by a liver-specific factor and is important for the liver specificity of EnhI. Our results further indicate that E and NF1 sites can increase the enhancer activity by 10-fold and that the E site is also essential for X promoter activity.

### MATERIALS AND METHODS

**Cell lines.** Huh7 is a well-differentiated human hepatoma cell line. This cell line was maintained in a 1:1 mixture of Dulbecco's modified essential medium and F12 medium supplemented with 5% fetal bovine serum (FBS). For preparation of the nuclear extracts, cells were grown in this nutrient mixture supplemented with 1.5% FBS and 7% newborn calf serum. CV1 is a monkey kidney cell line, and U87-MG is a neuroblastoma cell line. Both cell lines were maintained in Dulbecco's modified essential medium supplemented with 5% FBS.

**Preparation of nuclear extracts.** Nuclear extracts were prepared from 10 roller bottles of Huh7 cells by the method of Graves et al. (9). The protein concentration of the crude nuclear extract prepared by this method was between 3 and 5 mg/ml. For heparin-agarose column (Bethesda Research Laboratories) fractionation, the column was first equilibrated in TGMECK (25 mM Tris-HCl [pH 8.0], 10% [vol/vol] glycerol, 5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 1 mM dithiothreitol, 100 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride). The crude nuclear extract loaded on the column in TGMECK was stepwise eluted in TGMECK containing 100

\* Corresponding author.

mM (flow through fraction), 200 mM, 400 mM, and 600 mM KCl. The eluates were aliquoted and stored at  $-70^{\circ}\text{C}$  until use.

**EMSA and methylation interference experiments.** The electrophoretic mobility shift assay (EMSA) was carried out in a final volume of 20  $\mu\text{l}$ . The reaction mixture, containing 2.5  $\mu\text{g}$  of poly(d-I-dC) (Boehringer Mannheim), 50 mM KCl, 10 mM Tris-HCl (pH 8), 5 mM  $\text{MgCl}_2$ , 1 mM EDTA, 1 mM dithiothreitol, and 1 to 5  $\mu\text{l}$  of the fractionated nuclear extract, was incubated on ice for 10 min. After the addition of approximately 0.05 pmol of  $^{32}\text{P}$ -end-labeled DNA probe, the reaction was carried out at room temperature for another 20 min. The reaction mixture was then loaded directly onto a 4% acrylamide gel and electrophoresed in a low-ionic-strength buffer containing 6.7 mM Tris-HCl (pH 8.0), 3.3 mM sodium acetate, and 1 mM EDTA.

For methylation interference experiments,  $^{32}\text{P}$ -end-labeled DNA probe, methylated with dimethylsulfate (17), was used for the binding reaction as described above for EMSA. DNA probe bound by protein factors was purified on a preparative low-ionic-strength gel, eluted in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA–0.2 M NaCl overnight, purified again on an Elutip-d column (Schleicher & Schuell), and finally cleaved with piperidine and analyzed on a sequencing gel.

**DNase I footprinting experiments.** DNase I footprinting experiments were performed as described by Galas and Schmitz (5); 15  $\mu\text{g}$  of Huh7 crude nuclear extract or 40  $\mu\text{g}$  of HeLa extract (a generous gift of Amy Lee and Takafumi Nakaki) was used for each reaction. The results were analyzed on a 6% sequencing gel.

**DNA plasmids and gene expression experiments.** pTKGH (18), an enhancerless DNA plasmid, was used as a reporter for analyzing the enhancer activities of various HBV DNA fragments. In this DNA plasmid, expression of the human growth hormone (HGH) sequence was regulated by the thymidine kinase (TK) promoter of herpes simplex virus. HBV DNA fragments (27), after ligation to a *Hind*III linker, were cloned into pTKGH at the unique *Hind*III site located upstream of the TK promoter. The enhancer activities of various HBV DNA fragments were measured through the amount of HGH expressed by using a radioimmunoassay kit purchased from Nichols Instruments. In these transfection experiments, a control plasmid, pRSV-CAT (7), was cotransfected with the plasmid of interest into cells. pRSV-CAT expresses chloramphenicol acetyltransferase (CAT) and was used as an internal control for monitoring transfection efficiencies.

The promoterless plasmid pBR-CAT (29) was used as a reporter for analyzing the X promoter activities of various deletion mutants. This plasmid contains the CAT coding sequence. HBV DNA fragments were cloned into the unique *Hind*III site located upstream of the CAT coding sequence via *Hind*III linker. The promoter activities of various deletion mutants were measured through the amount of CAT expressed by using the method of Gorman (6). Similarly, in these transfection experiments, the control plasmid pTKGH (18), which expresses HGH, was used for monitoring transfection efficiencies.

DNA plasmids, purified by CsCl density gradients, were transfected into cells by the  $\text{CaPO}_4$  precipitation method (8). Forty-eight hours after transfection, the medium was collected for HGH assays and the cells were lysed for CAT assays. For each DNA plasmid, more than five different transfection experiments were routinely performed. The results presented in the figures represent the averages of the results of multiple transfection experiments.

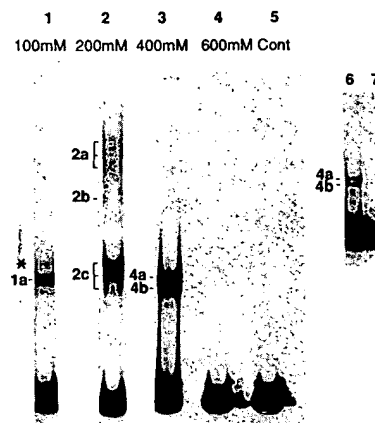


FIG. 1. EMSA for protein factors binding to the HBV EnhI enhancer-X promoter complex. The 120-bp *Stu*I-*Sph*I HBV DNA fragment (nucleotides 1115 to 1235 [27]) containing the EnhI enhancer and X promoter activities was subcloned into the *Sma*I and *Sph*I sites of the pUC18 vector. The HBV DNA fragment, reisolated from the resulting plasmid by using the flanking *Eco*RI and *Hind*III sites, was end labeled with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  and used as the probe for the assays. Lanes: 1, 100 mM KCl (flowthrough) fraction of the nuclear extract; 2, 200 mM KCl fraction of the nuclear extract; 3 and 6, 400 mM KCl fraction; lane 4, 600 mM KCl fraction; 5 and 7, no extract added. Cont, control sample. The band seen in lane 3 was actually a mixture of two bands. These two bands were clearly resolved in a separate experiment (lane 6). The asterisk denotes the band caused by cross-contamination of protein factors from the 200 mM KCl fraction. This band was not detected in other mobility shift experiments. The signal of band 2b is weak but is reproducible in different experiments.

## RESULTS

**Characterization of protein factor binding sites in the EnhI enhancer-X promoter complex.** As a first step in characterizing the HBV EnhI enhancer-X promoter complex, we decided to identify sequences that may be involved in regulating EnhI enhancer and X promoter activities. In our preliminary experiments, a 240-bp *Stu*I-*Tha*I DNA fragment containing sequences flanking the transcription initiation sites of the X mRNA were used for DNase I footprinting experiments using nuclear extract prepared from Huh7 cells, a well-differentiated human hepatoma cell line. In those preliminary experiments, all of the protein factor binding sites were found in a 120-bp region between the *Stu*I and *Sph*I sites located upstream of the X mRNA transcription initiation sites (see below and Fig. 3 and 5). Thus, this 120-bp DNA fragment was isolated and used for further analysis.

The crude nuclear extract prepared from Huh7 cells was loaded on a heparin-agarose column and stepwise eluted with 100 mM (flowthrough fraction), 200 mM, 400 mM, and 600 mM KCl solutions. The fractionated Huh7 nuclear extract was then incubated with the 120-bp EnhI enhancer DNA fragment for EMSA. As shown in Fig. 1, one major shifted DNA band was detected if the 120-bp EnhI DNA probe was incubated with the 100 mM KCl nuclear extract (lane 1, band 1a). The minor band denoted by an asterisk is an artifact possibly due to cross-contamination of factors of the 200 mM KCl fraction. This minor band was not detected in similar experiments. Multiple shifted DNA bands (lane 2, bands 2a, 2b, and 2c) were detected if the 200 mM KCl fraction of the nuclear extract was used. Two major shifted bands were detected with the 400 mM KCl fraction of the nuclear extract eluted (lanes 3 and 6, bands 4a and 4b). No

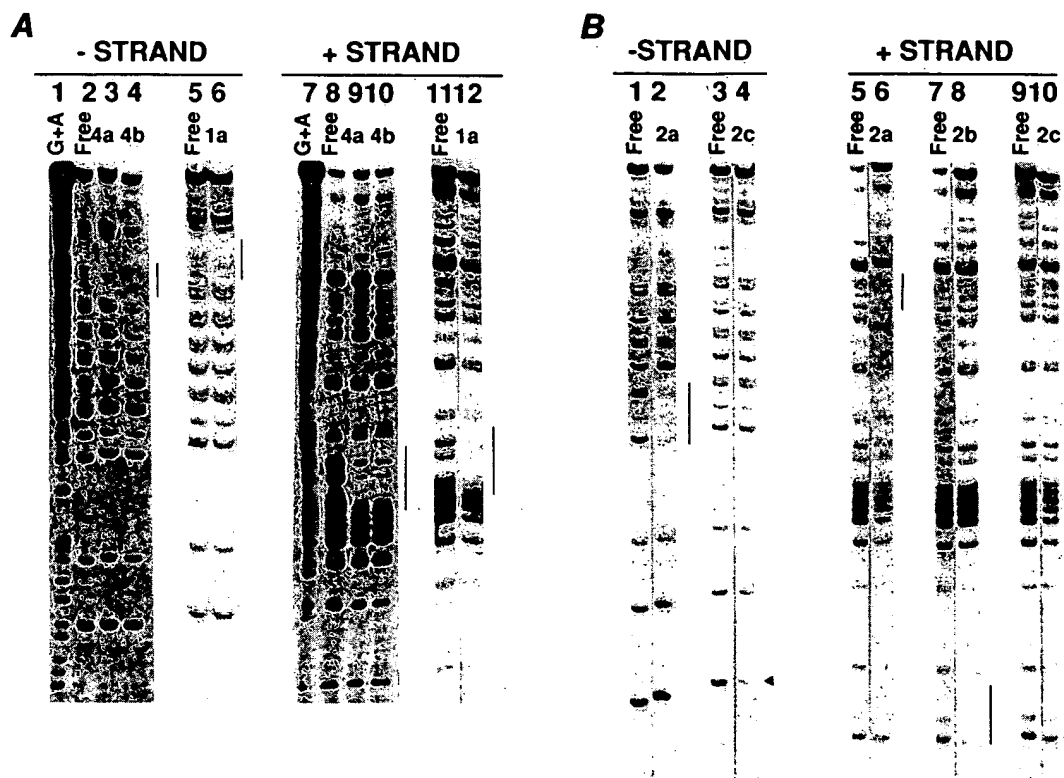


FIG. 2. Identification of protein factor binding sites in the HBV EnhI enhancer-X promoter complex. Details of the procedures are described in Materials and Methods. Lanes: G+A, G and A sequencing ladders; Free, free DNA probe; 1a, 2a, 2b, 2c, 4a, and 4b, the respective shifted bands identified in Fig. 1. Because of the difficulty of obtaining a sufficient amount of radioactivity, the methylation interference experiment was conducted on only the plus strand of band 2b. Bars and arrowheads indicate the locations of protein factor binding regions.

shifted band was detected with the 600 mM KCl fraction of the nuclear extract. The detection of multiple shifted bands indicates that multiple protein factors can bind to this 120-bp DNA fragment.

To determine the protein factor binding sites in the 120-bp DNA fragment, we performed methylation interference experiments. As shown in Fig. 2A, the protein factor binding sites of bands 1a, 4a, and 4b are identical and are located on the E site previously identified by DNase I footprinting experiments (1, 15). Since it has been shown that several protein factors, including C/EBP, AP1, and CREB, can bind to this site (4), bands 1a, 4a, and 4b (Fig. 1) may be bound by different protein factors.

Shifted DNA bands marked by 2a were bound by protein factors at the EP site previously detected by DNase I footprinting experiments (1) (Fig. 2B, lanes 2 and 6). This site contains a set of inverted repeats and was suspected to be bound by the ubiquitous dimeric EF-C factor (2). Because of clustered electrophoretic mobilities, 2a bands were likely produced as a result of protein modification or degradation.

The shifted band 2b was bound by a protein factor at the NF1 site. The signal of this band as shown in Fig. 1 is weak but reproducible, possibly as a result of the low concentration or the instability of this protein factor in Huh7 cells.

DNA bands marked 2c have the same protein factor binding site (Fig. 2B, lanes 4 and 10). Interestingly, this protein factor binding site is a new site and was not previously identified. Similar to 2a bands, because of clustered electrophoretic mobilities, the multiple 2c bands detected as

shown in Fig. 1 could be the result of protein modification or degradation.

Thus, there are four protein factor binding sites in the 120-bp region located upstream of the X mRNA transcription initiation sites. These four protein factor binding sites likely regulate the EnhI enhancer and X promoter activities. The locations of these four protein factor binding sites are shown in Fig. 3.

**Identification of a basal enhancer module in the EnhI-X promoter complex.** To examine the importance of these four protein factor binding sites for EnhI enhancer activity, we performed deletion mapping experiments. The DNA plasmid pTKGH, which contains the HGH coding sequence under the expression control of the herpesvirus TK promoter, was used as a reporter. The enhancer activities of various HBV DNA fragments were measured through the amount of HGH expressed. As shown in Fig. 4, the 120-bp *StuI-SphI* DNA fragment contains a strong liver-specific enhancer activity. It can activate TK promoter by approximately 50- or 90-fold, depending on the orientation, in liver-derived Huh7 cells but has only a marginal 2-fold activity in non-liver-derived CV1 and U87 cells. Inclusion of sequences flanking this 120-bp DNA fragment did not significantly increase the liver-specific enhancer activity (data not shown).

Deletion of the NF1 (2b) site reduces the enhancer activity by about 30% in liver cells to about 38- or 62-fold, depending on the orientation of the enhancer DNA fragment. Further deletion of the E (1a, 4a, and 4b) site led to a further decrease of the enhancer activity to about 10% of the original level.

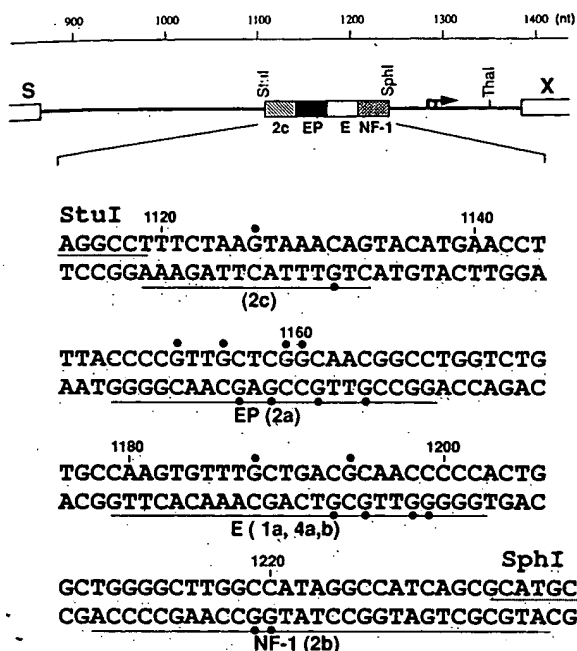


FIG. 3. Protein factor binding sites in the HBV EnhI enhancer-X promoter complex. Arrow indicates the transcription initiation sites of the X mRNA (10, 25). Dots indicate the locations of G residues which interfere with protein binding after methylation. Lines indicate the binding sites determined by DNase I footprinting experiments as shown in Fig. 5. The boundary between the E and EP sites was arbitrarily defined since the DNase I footprinting experiments shown in Fig. 5 revealed no clear separation between these two sites. nt, nucleotides; S and X represent the coding regions of the S and X genes, respectively.

Further deletion of the EP (2a) site led to the complete loss of the enhancer activity. Deletion of the 2c site alone also resulted in the loss of liver specificity and reduced the enhancer activity to a marginal two- to threefold. Further deletion of the EP site led to a further reduction of the enhancer activity to the background level. Thus, the 2c site is essential for the liver specificity, and it together with the EP site constitute a liver-specific basal enhancer module. Therefore, in contrast to previous belief (2), the downstream E site is not essential for the basal enhancer activity. The E site and the downstream NF1 site, however, can successively increase EnhI enhancer activity by a total of about 10-fold. These two sites thus are accessory modules of the EnhI enhancer element.

The 2c site is likely bound by a liver-specific protein factor. The observation that the 2c site is essential for the liver-specific activity of the EnhI enhancer and can constitute a liver-specific basal enhancer module with the EP site suggests that it may be bound by a liver-specific factor. To investigate this possibility, we performed DNase I footprinting experiments using nuclear extracts prepared from Huh7 hepatoma cells and HeLa cervical carcinoma cells.

As shown in Fig. 5, the EP, E, and NF1 sites are detected by nuclear extracts prepared from both Huh7 and HeLa cells. On the other hand, the 2c site is detected only with nuclear extract prepared from liver-derived Huh7 cells. Thus, this result supports the speculation that the 2c site is bound by a liver-specific factor.

Position-dependent interaction between the basal enhancer module and the downstream accessory modules. To under-

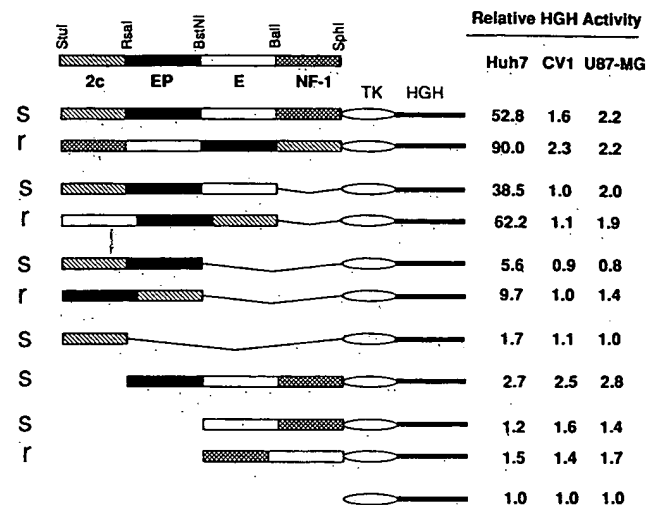


FIG. 4. Deletion analysis of the HBV EnhI enhancer element. Various HBV DNA fragments isolated by using the convenient restriction enzyme sites indicated were ligated to a HindIII linker and cloned into the unique HindIII site of the plasmid pTKGH (18) in front of the TK promoter. The amounts of HGH expressed by various HBV DNA constructs were normalized against that of the parental plasmid pTKGH, which expressed approximately 1 ng of HGH per ml in the medium of Huh7 cells and 5 ng/ml in the medium of either CV1 or U87 cells 48 h after transfection. s, sense orientation; r, reversed orientation.

stand how the basal enhancer module interacts with the downstream accessory modules, we translocated the downstream accessory modules containing E and NF1 sites to the upstream region of the basal enhancer module or inverted the polarity of the downstream accessory modules. As shown in Fig. 6, this translocation (pTL) or inversion (pDI) reduced the enhancer activity by approximately sixfold to

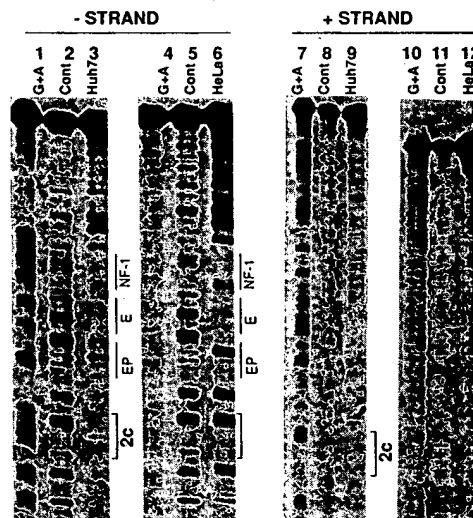


FIG. 5. Liver specificity of the protein factor binding to the 2c site. The NF1, EP, and E sites, as previously determined by Ben-Levy et al. (1) and Patel et al. (15), are indicated by lines. The 2c factor binding site is indicated by a bracket. The 240-bp StuI-Thal fragment containing the EnhI enhancer, the X promoter, and most of the 5' untranslated region of the X mRNA was used for DNase I footprinting experiments. Cont, control, no nuclear extract added.

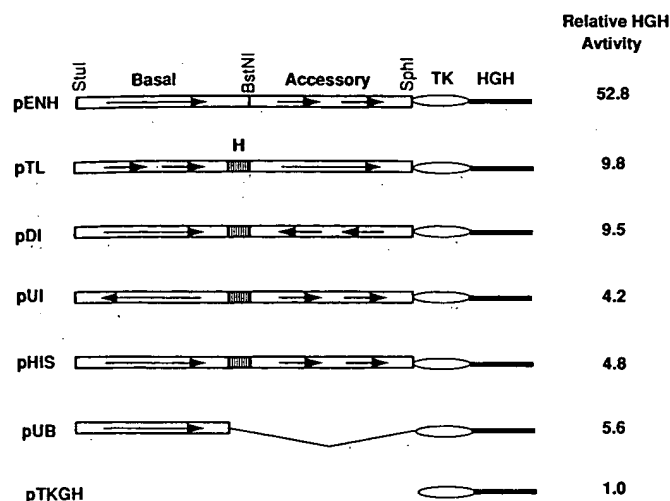


FIG. 6. Interaction between the basal enhancer module and accessory modules. Box with one arrow, basal enhancer module; box with two arrows, accessory module; hatched box, *HindIII* linker. The 120-bp *SmaI-SphI* HBV EnhI enhancer element was digested with *BstNI* for separation of the basal enhancer module and the accessory modules. The resulting two DNA fragments were ligated to a *HindIII* linker and recloned into the *HindIII* site of the pTKGH vector. The polarities of the inserts in the vector were determined by direct sequencing. pENH, plasmid containing the wild-type 120-bp EnhI DNA fragment; pTL, translocation of the accessory modules to the upstream region of the basal enhancer module; pDI, inversion of the polarity of the downstream accessory module; pUI, inversion of the polarity of the upstream basal enhancer module; pHIS, insertion of 11 bp between the basal enhancer module and the accessory modules; pUB, upstream basal module only. As for Fig. 4, the amounts of HGH expressed by various HBV DNA constructs were normalized against that of the parental plasmid pTKGH.

slightly above the activity of the basal enhancer module. Inverting the polarity of the upstream basal enhancer module also significantly reduced the enhancer activity to about the basal enhancer module level. Interestingly, inserting 11 bp, approximately a helix turn, between the upstream basal enhancer module and the downstream accessory modules also reduced the enhancer activity to about the basal level. These results indicate that the downstream accessory modules have to interact with the upstream basal enhancer module in an orientation- and distance-dependent manner to increase the enhancer activity.

The E site of EnhI is an essential component of the X promoter. The importance of the four protein factor binding sites on the regulation of X promoter activity was also analyzed by deletion mapping experiments using the promoterless plasmid pBR-CAT (29) as the reporter. The X promoter activities of the various HBV DNA fragments were measured through the amount of CAT expressed. As shown in Fig. 7, deletion of the 2c site reduced X promoter activity by about twofold in Huh7 cells but had no effect in non-liver-derived cells. Since the 2c site is essential for the liver-specific activity of the basal enhancer module, the reduction of X promoter activity in liver cells is likely due to loss of the liver-specific basal enhancer activity. This could explain why there was no reduction of X promoter activity in non-liver-derived CV1 cells. Further deletion of the EP site led to a reduction of X promoter activity by slightly less than twofold in both liver and non-liver cells. Similarly, this slight

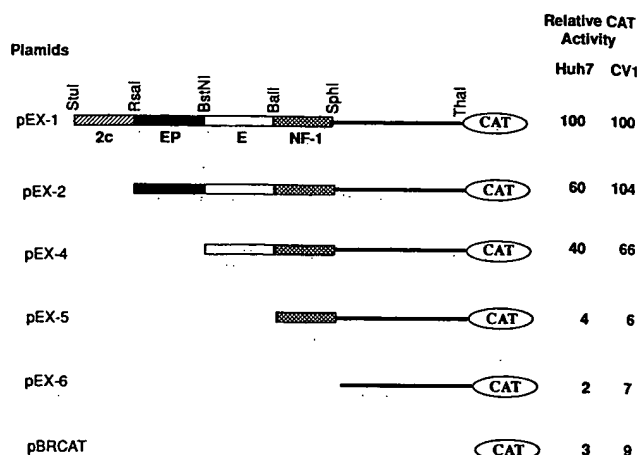


FIG. 7. Deletion analysis of the X promoter. HBV DNA fragments digested by *SmaI* at the 3' end and various restriction enzymes at the 5' end as indicated were ligated to a *HindIII* linker and cloned into the unique *HindIII* site of pBR-CAT in front of the CAT coding sequences. The amounts of CAT expressed by various HBV DNA constructs were normalized against that of pEX-1, which gave rise to approximately 80 and 30% CAT conversions for Huh7 and CV1 cells, respectively.

reduction could be due to the loss of the weak ubiquitous enhancer activity constituted by the EP, E, and NF1 sites (Fig. 4). Further deletion of the E site reduced X promoter activity to the background level in both liver and non-liver cells. Thus, the E site is an essential component of the X promoter and it has dual functions: to regulate the X promoter activity and to increase the activity of the basal enhancer module constituted by the upstream 2c and EP sites.

## DISCUSSION

In this report, we have identified four protein factor binding sites clustered in a 120-bp region upstream of the X mRNA transcription initiation sites (Fig. 3). These four sites are involved in regulation of the HBV EnhI enhancer and X promoter activities.

Among the four protein factor binding sites, the 2c site is essential for the liver specificity of the enhancer element. This site together with the juxtaposed EP site constitute a liver-specific basal enhancer module (Fig. 4). This module can activate the herpesvirus TK promoter by 5- or 10-fold, depending on the orientation, in liver cells. DNase I footprinting experiments (Fig. 5) indicate that the 2c site is likely bound by a liver-specific factor. This result could explain why the 2c site is essential for the liver specificity of the EnhI enhancer element. Recently, Patel et al. (15) have also identified a protein factor binding site which partially overlaps the 2c site. This site, which they called the FPV site, was detected when nuclear extract prepared from rat liver was used for DNase I footprinting experiments. This site was not detected with nuclear extract prepared from rat spleen. Thus, the FPV site may also be occupied by a liver-specific factor. The FPV site was not detected in our methylation interference or DNase I footprinting experiments. It is likely that these two sites were in fact bound by the related human and rat protein factors, respectively. The difference between the binding sites mapped by Patel et al.

(15) and us (Fig. 3) could be caused by subtle structural differences between the human and rat protein factors.

In contrast to previous belief, the downstream E site is not essential for the basal enhancer activity. On the other hand, this site and the downstream NF1 site can successively increase the activity of the basal enhancer module by approximately another 10-fold (Fig. 4). Thus, the E and NF1 sites are accessory modules of the EnhI enhancer element. The observation that the interaction between the basal enhancer module and the two downstream accessory sites is orientation and distance dependent (Fig. 6) indicates that protein factors binding to these modules must interact with each other in a position-dependent manner to activate the transcription complex binding to the downstream promoter element. It is interesting to note that in methylation interference experiments (Fig. 2), E (1a, 4a, and 4b) and EP (2a) sites were clearly distinguishable, but in DNase I footprinting experiments, there is no clear separation between the E and EP sites (Fig. 5). Ben-Levy et al. (1) have suggested that this junction region may be bound by NF1, a protein factor involved in gene transcription and adenovirus DNA replication (12). It is likely that NF1 mediates the interaction between the basal enhancer module and the accessory modules and that this process is orientation and distance dependent. Further experiments will be needed to verify this hypothesis.

The E site is essential for X promoter activity (Fig. 7). This site can be bound by several protein factors, including C/EBP, AP1, and CREB (2; Fig. 1 and 2). Because this site also has an accessory function for the enhancer activity (Fig. 7), competition among protein factors for binding to this site may be an important way for HBV to regulate EnhI enhancer and X promoter activities. In this regard, it is interesting to note that C/EBP down-regulates (16) and AP1 up-regulates (3) EnhI enhancer activity. Since EnhI can increase the downstream C promoter activity by about 60-fold (unpublished observation) and has only a marginal 2- to 3-fold activity on the X promoter (Fig. 7), this type of regulation can lead to the differential activation of different HBV genes and may be important for replication of the virus.

#### ACKNOWLEDGMENTS

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